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MINI-REVIEW

Spatial transcriptomics and proteomics technologies for deconvoluting the tumor microenvironment

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Abstract

The tumor microenvironment (TME) harbors heterogeneous contents and plays critical roles in tumorigenesis, metastasis, and drug resistance. Therefore, the deconvolution of the TME becomes increasingly essential to every aspect of cancer research and treatment. Novel spatially-resolved high-plex molecular profiling technologies have been emerging rapidly as powerful tools to obtain in-depth understanding from TME perspectives due to their capacity to allow high-plex protein and RNA profiling while keeping valuable spatial information. Based on our practical experience, we review a variety of available spatial proteogenomic technologies, including 10X Visium, GeoMx Digital Spatial Profiler (DSP), cyclic immunofluorescence-based CODEX and Multi-Omyx, mass spectrometry (MS)-based imaging mass spectrometry (IMS) and multiplex ion-beam imaging (MIBI). We also discuss FISSEQ, MERFISH, Slide-seq, and HDST, some of which may become commercially available in the near future. In particular, with our experience, we elaborate on DSP for spatial proteogenomic profiling and discuss its unique features designed for immuno-oncology and propose anticipation towards its future direction. The emerging spatially technologies are rapidly reshaping the magnitude of our understanding of the TME.

KEYWORDS
DSP, multi-omics, spatially-resolved, tumor microenvironment TME

1 | INTRODUCTION

With the surge of immunotherapy for treating multiple tumors in basic research and clinical oncology, resolving the complexity of the tumor microenvironment (TME) has become the central obstacle to disentangle ill-defined drug resistance mechanisms and to facilitate biomarker-driven drug discovery for patient stratification. Immune checkpoint modulation has proven its non-discriminatory potency and unprecedented durability.[1–3] Cell therapies, including chimeric antigen receptor T cells therapy (CAR-T), have demonstrated sustained responses for most patients.[4] Notwithstanding the success made in a few indications, a large portion of patients is still plagued with undesired responses, cumulative adverse effects, and refractory diseases.[3,5] More disappointingly, cell therapies are now in a huge dilemma in treating non-hematologic tumors either due to the challenge of finding highly cancer cell-specific antigens or limited drug trafficking within the immune-suppressive TME.

In drug development, much attention has been drawn to improve the efficacy using combination strategies to overcome resistance and expand administrable indications under appropriate biomarker guidance.[3,6,7] In solid tumors, the intricate biological mechanisms associated with heterogeneity reside in the TME where regulatory molecules are constitutively interacting with the microenvironment. This ultra-complex system harbors dynamic cellular cross-talk via direct contact, indirect extracellular signaling, and/or extracellular vesicles (EV). Secretome within extracellular matrices, mainly consisting of cytokines, growth factors, hormones, enzymes, and EV, acts as mediators during tumorigenesis, causing drug resistance via multifaceted mechanisms.[3,8] Therefore, exploring the dynamic interplay within the TME is indispensable for elucidating mechanisms

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permitting effectual drug discovery and biomarker implementation. Though PD-L1 expression and microsatellite instability (MSI) demonstrated their predictive biomarkers efficacy for multiple cancers, they still show limited capability for desirable stratification. Single-cell technologies have provided an unprecedented analytical scope in elucidating tumor heterogeneity and uncovering complex immune contexture during oncogenesis.[9] Spatial biological technologies add an additional layer of spatial information to TME studies. Besides, understanding the spatiotemporal information is paramount in deciphering key mechanisms of interplay between tumor cells and immune contexts. Hypothetically, TME can be spatially divided into three classes: "Inflamed" (positive immunogenic TME), "immune deserts" (lack of immunogenic TME), and "immune excluded" (predisposed to immunogenic TME).[3,10] Furthermore, tumor immunity in the microenvironment (TIME) states that functional PD1/PD-L1 relies upon not only the expression but also the co-localized leukocytes. Only by triggering immune-primed tumor cells within the TME could tumor-infiltrating lymphocytes (TILs) evoke an effective and controlled immunogenic response, consequently leading to favorable antitumor effects.[11,12] These concepts, together with our handful of knowledge in explaining the complex mechanisms attributable to the dynamic regulation of the TME render us to pose utter needs for an in-depth understanding of the TME.

Despite strong enthusiasm in finding novel therapeutic targets, combinatory immunotherapy has outpaced monotherapy in clinical trials with the top-tier focusing on checkpoint inhibitors with VEGF/VEGFR and chemotherapy.[6] Other combinations with druggable proteins, cancer vaccines, and oncolytic viruses are also ongoing. For adoptive therapies, though TIL infusion and CAR-T have delivered fruition on hematopoietic malignancies, inducing a harmonized immunogenic microenvironment is still challenging.

Three major aspects currently under thorough investigation are (1) to find the Achilles heel of tumors in particular biological contexts aiming to increase on-target specificity; (2) to keep a manageable balance of on-target/off-target toxicities by modifying immunogenicity in TME; (3) to discover the rationale monotherapies and combinatory strategies under robust biomarker guidance.[13–15] To gain deeper insight, advanced technologies are needed in addressing an array of key questions thereof. In this mini-review, we summarize frontier spatial technologies for TME exploration. We also share our extensive experience on Digital Spatial Profiling (DSP) technology and elaborate on its unique features to analyze both protein and RNA, anticipating its broad application from TME perspectives.[16]

2 | SPATIAL TRANSCRIPTOMICS TECHNOLOGIES

2.1 | Spatial transcriptomics technologies not yet commercially available

For imaging-based methods, FISSEQ (fluorescent in situ sequencing) and MERFISH (multiplexed error-robust fluorescence in-situ hybridization) both emerged in recent years (Table 1 and Figure 1). FISSEQ converts RNA molecules to cDNA fragments in-situ, forming a circularized template catalyzed by CircLigase to allow rolling cycle amplification. Amplicons are cross-linked and sequenced via SOLiD sequencing using confocal imaging to allow fluorescence-guided interpretation of target molecules in space.[17] FISSEQ enables subcellular interrogation of RNA species with theoretical limits over 16,000 genes, though technically challenging due to signal crowding at heavy computational expenses. Although compatible with formalin-fixed paraffin-embedded (FFPE) tissues, scannable areas are confined within 4×4 mm regions, and application evidence is sparse as sophisticated workflows are impractical across laboratories. Sequencing biases are also unavoidable with predisposed rRNA and active RNA enrichment.[17] Similarly, MERFISH combines in-situ hybridization and imaging techniques with a modified 4 hamming distance (MHD4) designed within encoding probes to increase the target calling with reduced misidentification errors.[18] MERFISH applies four sets of probes with total sequence coverage up to 192 bp for a single transcript and overhanging sequences for fluorescence probe anchoring. Sequential images are acquired with a target-specific 4-color decoded readout pattern presented in binary formats.[18] Alternative coding designs allow MERFISH to detect over 10,000 transcripts; however, its applicability for tissue specimens is still limiting.[19] Besides, such methodology is yet widely inaccessible, preventing it from cross-lab validation. Another evolving technology employs spatial barcoding techniques and is well featured by Slide-seq and high-definition spatial transcriptomics (HDST).[20,21] Slide-seq deposits DNA-barcoded and micrometer-sized beads into microwells, forming a one-bead one-well monolayer on glass substrates, and beads conjugated with spatial indexing barcodes allow spatial mapping via SOLiD sequencing.[21] RNA from cryosected tissues is released and captured via polyA tails in-situ, followed by reverse transcription and sequencing.[21] Similarly, HDST has higher resolutions in space (2-μm) for depositing beads with barcoded-oligos into hexagonal-shaped wells across substrates.[20] Spatial decoding is performed via multiple rounds of scanning using tri-fluorescence space decoding oligos to enable $\log_3 N$ number of spatial indexing.[20] RNA released from hematoxylin & eosin (H&E) stained frozen tissue sections is captured via poly(d)T overhangs and reverse-transcribed, and the product barcode-containing sequences are cleaved, sequenced, and mapped back in space coordinate to complete the high-plex profiling.[20] Though both methods only emerged recently with a handful of data available, advantages of over 10,000 measurements, high-plexity, and single-cell or sub-cellular detection capacity likely facilitate their broad application in TME-driven research.

2.2 | Commercially available spatial transcriptomics technologies

Recently, two spatial transcriptomic techniques have become commercially available. Visium spatial transcriptomics (ST) is a kit-based assay requiring much less sophisticated processes than HDST. Similarly, with a loose design of spatial matrix (55 μm wide), tissues can be placed, fixed, and H&E stained for bright-field imaging followed by

TABLE 1 Key features of spatial technologies for RNA/protein profiling

	Basic principles	Analytical resolution	Compatible tissue type	High-plexity	Laboratory procedures	De-novo/ targeted	TME analysis driven	Scan area size	Sample destructiveness	Sample practical throughput	Quantitative capacity
Transcriptomics											
DSP	UV-cleaved oligo-conjugated RNA probes and barcodes counting or NGS readout	60-100 cells	FFPE, TMA and fresh-frozen samples	84 (Panelized)/ 1833(CTA)/18,(	Kit-based procedure/ Automated process/Cross-lab availability	Targeted	Purposely designed	14.6 mm x 36.2 mm	Non-destructive	8 in 3 days	Semi-quantitative
10X Visium	RNA capturing through oligo-dT overhangs and RNA-seq	1-10 cells	Fresh-frozen samples	Theoretical whole transcriptome	Kit-based procedure/ Labor intensive with multiple manual steps	De-novo	Not specifically	6.5 mm x 6.5 mm	Destructive	4-8 in 5 days	Semi-quantitative
Slide-seq	Spatial barcoded beads and SoLiD-seq	10 μm	Fresh-frozen samples	Over 10,000	Sophisticated hardware and complicated imaging based sequencing procedure	De-novo	Not specifically	μm range	Destructive	2 weeks +	Semi-quantitative
HDST	Microwell-based fluorescence spatial indexing beads and RNA-seq	2 μm	Fresh-frozen samples	Over 10,000	Sophisticated hardware	De-novo	Not specifically	5.7 mm x 2.4 mm	Destructive	1-2 weeks	Semi-quantitative
MERFISH	Interative Imaging of MHD4 in-situ hybridization	Sub-cellular	Fresh-frozen samples	Over 10,000	Iterative process with multiple imaging acquisition and computation	Targeted	Not specifically	22 diameter (theoretical)	Destructive	1 in 5 days	Semi-quantitative

(Continues)

TABLE 1 (Continued)

	Basic principles	Analytical resolution	Compatible tissue type	High-plexity	Laboratory procedures	De-novo/ targeted	TME analysis driven	Scan area size	Sample destructiveness	Sample practical throughput	Quantitative capacity
FISSEQ	Template circularization, RCA and SOLiD-seq	Sub-cellular	FFPE/Fresh-frozen samples	16,000	Multiple amplification and imaging based sequencing procedure	De-novo	Not specifically	4 mm x 4 mm	Non-destructive	2 weeks +	Semi-quantitative
Proteomics											
DSP	UV-cleaved oligo-conjugated primary antibodies and barcodes counting	Five cells	FFPE, TMA and fresh-frozen samples	90	Kit-based procedure/ Automated process/Cross-lab availability	Targeted	Purposely designed	14.6 mm x 36.2 mm	Non-destructive	8 in 3 days	Semi-quantitative
Multi-Omyx	Cyclic immunofluo-rescence	Single cell	FFPE and fresh-frozen samples	Over 60	Standardized workflows	Targeted	Purposely designed	45 mm x 20 mm	Non-destructive	1-2 in 3 days	Semi-quantitative
CODEX	Antibodies with cyclic oligo-barcoded reporter	Single cell	FFPE and fresh-frozen samples	50	Standardized workflows with automation	Targeted	Purposely designed	4 mm x 4 mm	Non-destructive	5 in 5 days	Semi-quantitative
MIBI	Antibody isotope labeling with MALDI-MS	Sub-cellular	FFPE and TMA	100	Heavy instrumentation and standard workflows	Targeted	Purposely designed	No larger than slides	Non-destructive	5-10 in 3 days	Semi-quantitative
MALDI-IMS	MALDI-IMS	75 μm	FFPE and fresh-frozen samples	50-100 peptides	Heavy instrumentation and non-standardized workflows	De-novo	Not specifically	No larger than slides	Destructive	5-10 in 3 days	Semi-quantitative

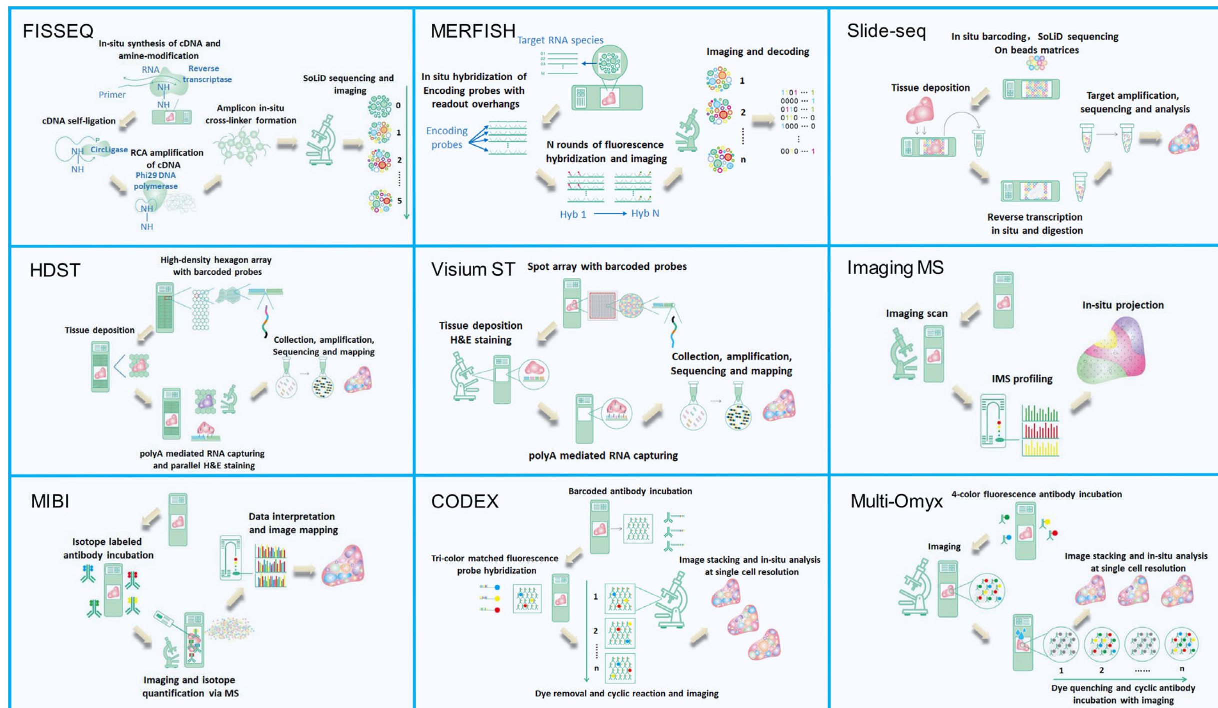

FIGURE 1 Schematic depiction of spatial transcriptomics and proteomics technologies: individual figures represent their basic principles and workflows

permeabilization to allow RNA capturing through oligo-dT overhangs. Hybridized sequences are then reverse-transcribed for sequencing. Resultant data are mapped back to spatially arranged spots allowing de-novo quantification of RNA species.[22] This technology does not need heavy instrumentation. The major prerequisites for hardware include cryosection stations, standard fluorescence microscopies, and PCR facilities. Visium ST has already been hinted for TME-related research in multiple tumors.[23] Despite attempts made to revolutionize our view towards this technology, bottlenecks still exist. To precisely capture the desired samples, it requires experienced handling for cryosection, a crucial step that dramatically affects the results. Moreover, the matrix design allows only 6.5 × 6.5 mm space for tissue deposition limiting comprehensive exploration of the TME. Though the sub-histological resolution is achievable, loss of between-spot information may miss key information from rare cells. Tissue incompatibility with FFPE due to common RNA degradation is another drawback, although initial development showed some promising potential.[24]

To compensate for some drawbacks, DSP emerged as an alternative technology.[25] It combines a wet-lab protocol with an integrated system to perform regions-of-interest (ROIs) selection using fluorescence-guided methods (Figure 2). Typically, fluorophore-tagged antibodies recognizing different cellular compartments are used to obtain a morphological overview of tissues. Pre-incubated and oligo-conjugated RNA probes for corresponding targets are photocleaved via linkers, and the oligos are collected automatically for quantification via fluorescence barcode counting (via nCounter) or through NGS. The resultant data are mapped back to individual ROIs, reflecting in-situ expression.[25] The plexing capacity of DSP transcriptomics offers 3-tiers fixed panels (Table 1). Another unique advantage of DSP is its versatility for multiple tissue formats, including full FFPE and fresh frozen sections, tissue microarrays (TMA), and fixed cell culture. It is highly sensitive, with only 60–100 cells to yield usable data.[25] The morphological-driven ROI selection can also be achieved under conventional histological guidance on parallel slides (HE staining or IHC), offering a great advantage for TME analysis where different cellular contextures are known to play deterministic roles, including TILs, tumor-associated macrophages (TAMs), tertiary lymphoid structures (TLSs). These applications have already been used in resolving mechanisms for TLS in melanoma and in finding TAM-associated biomarkers in melanoma and NSCLC.[26,27] Despite those advantages, DSP bears certain limitations. ROI selection can be subjective, and though the targeted approach increases the detection sensitivity and quantification robustness, it loses the potential to discover novel RNA species. Near single-cell analysis is achievable, but for cellular type fractionation, computational deconvolution has to be adopted. However, with unprecedented advantages, its broad application in immuno-oncology is expected.

3 | SPATIAL PROTEOMICS TECHNOLOGIES

3.1 | Mass spectrometry-based spatial proteomics technologies

Imaging Mass Spectrometry (IMS) was developed two decades ago with proof-of-concept studies assessing its accuracy, multiplexing

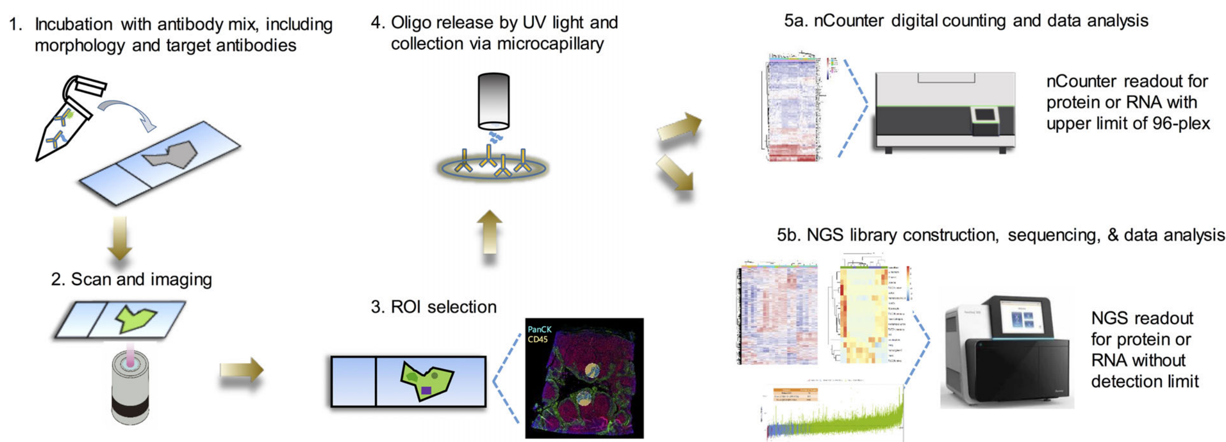

FIGURE 2 DSP workflow. 1. Tissue sections are deparaffinized, rehydrated, and morphologically stained together with a cocktail of antibodies or RNA probes conjugated with UV-cleavable oligos. 2. Digital scanning with up to four fluorescence markers simultaneously. 3. ROI selection with any defined contours. 4. UV cleavage for oligo collection for individual ROIs. 5a/b. Probe counting via nCounter or next-generation sequencing for quantification

capacity, and detectable peptide mass ranges.[28,29] A typical IMS incorporates a matrix-assisted laser desorption ionization mass spectrometer (MALDI-MS) with micrometer laser beams shed on tissues prepared on a matrix absorbed metal substrate. While ionized analytes from laser-excited spots are induced into the mass spectrometer for peptide identification, tissue images can be simultaneously generated via a raster scan generating coordinating data between spot location and associated mass spectrum.[30] As proteomic-driven, it is widely compatible with frozen and FFPE samples with a theoretical detectability of 50–100 peptides at a time.[31] It is a quantitative antibody-free approach without tissue destruction, making it compatible with downstream applications such as digital PCR.[32] Highlights of MALDI-IMS in multiple solid tumors have demonstrated in resolving the complexity of spatial patterns of proteins in heterogenous tissue specimen, most of which focused on tumor typing, grading, and sub-histological classification.[33,34] However, heavy instrumentation and non-standardized workflows hinder its wider application. Besides, obtaining higher resolutions for TME still proves to be difficult due to its untargeted detection nature and sub-optimal quantification capability.[31] The latter defect was partially overcome by multiplexed ion beam imaging (MIBI). MIBI uses a mixture of elemental isotope-labeled primary antibodies to react with tissues mounted on conductive substrates.[35] A rasterized oxygen duoplasmatron surface ion beam is applied to liberate antibody-bound isotopes that are then detected via MS as secondary ions. Data are then merged with single-cell resolution images to obtain a tabulated chart representing spatial profiles.[35] Technically MIBI can interrogate up to 100 TME-related targets in one experiment making it a superior tool for immuno-oncology research. Its applications in a few solid tumors have also been indicated.[36,37] Nevertheless, existing disadvantages, including its multiplexing capacity strictly limited to 100 and the requirement for expensive instrumentation and metal isotope-labeled antibodies, limits its extensive application.

3.2 | Antibody-based spatial proteomics technologies

Multiple antibody-based technologies have been emerging to provide amenable analytical power for TME profiling, with a cyclic multispectral signal generation being a major direction. Co-detection by indexing (CODEX), among several others, uses mixtures of indexable oligo-tagged antibodies for in-situ staining. Each cyclic reaction allows one set of spectrally separable reporter oligos to hybridize with their corresponding oligo-barcoded antibodies and is followed by image acquisition and reporter removal. This process is iterated to obtain consecutive multiparameter images for stacking, generating spatially informed expression profiles at a single-cell resolution.[38] Compatible with FFPE tissues, CODEX features hyper-plexing protein profiling (over 50 markers) in one slide. The microfluidic system allows an automated process for deeper TME profiling compared to its predecessors.[39,40] By analyzing 56 protein markers together on a colorectal cancer cohort, the immune TME landscapes have been depicted generating cellular neighborhoods to stratify patients at risk.[39] Drawbacks include scannable ranges within a 4 × 4 mm area limiting broad exploration of larger tissues and the requirement for tissue mounting onto coverslips as a pre-processing step. Another technology, Multi-Omyx, differs in that it repetitively stains tissues with fluorescent antibodies with consecutive imaging and non-destructive chemical quenching of fluorophores. Resultant image series are stacked and algorithm-aligned to provide single-cell spatial expression patterns.[41] Multi-Omyx is proven to allow over 60 markers co-profiling and suited for conventional FFPE samples. Using a 27-plex profiling, researchers explored breast carcinoma and defined 8 proteomic clusters based on 18-marker co-expression, and some are associated with glycolytic metabolism and clinical outcomes.[42] In a gastric cancer cohort, a set of 20-plex vascular and immune cell biomarkers revealed four stromal subtypes that likely have

fundamental biological characteristics associated with clinical outcomes and therapeutic efficacy.[43] Multi-Omyx supports customizable targets-of-interest selection. However, antibody combinations have to be validated thoroughly for sequential application as loss of immune affinity may occur when multiple cycles are applied.

Distinctively, DSP features its digital quantification by employing proprietary bead-on-string barcoded counting mechanisms, which vastly accelerates the workflow with a typical throughput of 12 samples for 90-plex protein assays in 3 days. It has been thoroughly cross-validated with quantitative immunofluorescence and IHC.[25] While not designed to address questions at single-cell levels, its distinct rare-cell function allows sub-population enrichment crucial for TME analysis.[25] Its ultra-sensitivity allows 5 cells for quantification with outstanding linearity and, with that in aid, refined expression patterns of TLSs in melanoma were elucidated.[44] In pancreatic cancer, by separating tumor, immune cells, and stroma regions for patients receiving different therapeutic combinations, specific treatment-related expressions were identified, warranting further biomarker investigation.[45] In uveal melanoma, paired pre-and post-treatment biopsies were analyzed, uncovering treatment-specific immune-alteration predictive outcomes.[46] Despite the early work, limitations still exist as to increase its plexing capacity and to circumvent the bottlenecks for single-cell profiling, thereby expanding data dimensionalities extractable from finite materials. Conclusively, we envisage a broader application context of these spatial proteomic technologies in translational research and clinical care.

4 | CONCLUSIVE REMARKS AND FUTURE PERSPECTIVES

In keeping pace with the needs in immuno-oncology research, high-dimensional spatial profiling will provide invaluable information. In light of the rapid progress in single-cell transcriptomics and proteomics to resolve tumor heterogeneity, projection of key information back at the spatial level is seemingly inevitable. It will fundamentally upscale the horizon of our understanding of TME. Although at their application infancy, with those cutting-edge analytical tools at hand, we hold optimistic opinions that the development of novel biomarkers for immunotherapeutic regimens, companion diagnostic tools for patient streatification, and mechanism elucidation for drug resistance can be dramatically accelerated.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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